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DEVELOPMENT OF A CRYPTOSPORIDIUM VACCINE

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Saskatchewan ADF Final Progress Report

"Development of a Cryptosporidium Vaccine"

submitted by:

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Objective

The overall objective for the project was to develop an effective Cryptosporium vaccine for use in cattle. In order to achieve this goal, we have proposed five specific aims over 3 years which need to proceed in a logical fashion as outlined below:

1) develop monoclonal antibodies to Cryptosporidia, 2) identify Cryptosporidia antigens that are involved in attaching to host cells, 3) identify genes encoding Cryptosporidia attachment antigens, 4) construct DNA plasmids containing Cryptosporidia specific genes, and 5) immunize animals with DNA plasmids containing Cryptosporidia specific genes.

Progress on Each Specific Aim (Milestones)

Specific Aim 1 – Development of Monoclonal Antibodies

One of the first approaches to developing a vaccine or any research project is to develop the reagents that are required. In vaccine development, one of the most critical components is to identify the particular antigen that will be used as a vaccine. Since infectious agents such as bacteria or parasites - Cryptosporidia contain thousands of proteins, it will be extremely time consuming to test each individual protein for its potential as a vaccine. Thus, one of the approaches that we have used is to develop monoclonal antibodies that could inhibit the attachment of the parasite to host cells. If we could prevent attachment of Cryptosporidia, the parasite would not enter cells and, therefore, would not initiate infection. Thus, the protein recognized by the monoclonal antibody would be an ideal protein for use as a vaccine. Therefore, to expedite the identification of the potential proteins that are involved in attachment of the cells, we attempted to develop monoclonal antibodies that would inhibit the attachment of Cryptosporidia to mammalian cells in culture.

To produce monoclonal antibodies, we needed to produce a stock of *Cryptosporidia parvum* antigen which could be used for immunizing mice. This was achieved by infecting neonatal calves with an isolate of *C. parvum*. The feces from infected calves was collected and stored in an equal volume of 2.5% potassium dichromate at 4°C. Oocyotes were isolated from feces by sucrose flotation centrifugation followed by ultracentrifugation in cesium chloride gradients. This allowed the isolation of pure oocytes. The purified oocytes were then stored at 4° for future use and production of monoclonal antibodies.

To produce monoclonal antibodies, mice were immunized intraperitoneally three times at four-week intervals with 50 mg of purified Cryptosporidium. They were boosted a final time and three days later the animals were sacrificed and the spleens were harvested. The individual spleen cells were fused to an immortalized partner (NS-1) using polyethelene glycol as a fusion agent. The hybridomas were cultured in 96 well plates and positive hybridomas were selected by enzyme immuno assays (ELISA) for clones that were producing antibodies to Cryptosporidia antigens. The hybridomas secreting large quantities of antibody were then subcloned to ensure that each of the individual clones was producing a single monoclonal antibody.

To further identify that the antibodies reacted with a single protein, Cryptosporidia antigens were resolved by electrophoresis, transferred to membranes and immunoblotted with the individual hybridoma fluids. Based on the ELISA and immunoblotting assays, a total of 12 individual monoclonal antibodies were isolated which reacted specifically with Cryptosporidia proteins.

Specific Aim 2 - Identification of Antigens Involved in Attaching to Host Cells

The monoclonal antibodies described above were incubated with *Cryptosporidia parvum* and tested for the ability to inhibit the attachment of Cryptosporidia to HT-29 cells. HT-29 cells were obtained from American Type Collection and are known to be susceptible to infection with *Cryptosporidia parvum*. To test for the ability of *Cryptosporidium parvum* to infect HT-29 cells, cells were cultured in Dulbecco's modified Eagles media plus glutamine supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere. The cells were grown in cover slips and when they were 80% confluent they were exposed to 5×10^5 sporocytes to measure invasion. Figure 1 demonstrates the inhibition of invasion of HT-29 cells by Cryptosporidia if incubated with monoclonal antibodies prior to addition to HT-29 cells. Two of the twelve monoclonal antibodies could inhibit the invasion of HT-27 cells. Although monoclonal antibody 222 appeared to inhibit slightly more efficiently than 1101 both of them could significantly reduce infection. None of the other monoclonal antibodies inhibited attachment to HT-29 cells (eg. 2F12). To determine whether the inhibition was antibody concentration dependent various concentrations of the monoclonal antibody were incubated with *Cryptosporidia parvum* prior to addition to HT-29 cells. Figure 2a demonstrates that at concentrations of 1 ug or higher there was significant inhibition with both monoclonal antibodies. Monoclonal antibody 2F12 served as a excellent control for protein concentration since it did not inhibit infectivity of *C. parvum.*

To further determine whether there was a synergistic effect between the two monoclonal antibodies capable of inhibiting attachment we conducted a subsequent series of experiments where individual antibodies were tested for their ability to inhibit *C. parvum* infection of cells or combinations of antibodies. Figure 2b clearly demonstrates that there is no synergistic or additive effect between the two different monoclonal antibodies. These results suggest that either both antibodies are directed against the same protein or that if they're directed against two different proteins these proteins interact with host cells independently.

Specific Aim 3 - Identify the Genes Encoding the Cryptosporidia Attachment Antigens

In order to identify the proteins recognized by the monoclonal antibodies we performed immunoblots directed against total, soluble Cryptosporidia proteins separated on a Western blot. Figure 3 shows that antibodies recognize a band migrating at 22,000 molecular weight. To identify the gene that corresponded to the protein recognized by the monoclonal antibodies we conducted a partial sequence of the protein to identify the nucleic acid sequence. Using this approach, we could then identify the gene encoding for the proteins recognized by monoclonal antibodies 222 and 1101. These antigens and genes were identified as AG-1 (monoclonal 1101) or AG-2 (recognized by monoclonal

antibodies 22). The gene sequence of each of these proteins is presented in Figures 4a and b. Using molecular biology techniques we cloned these antigens into a procaryotic expression vector (*E. coli)*. Following production of the protein in *E. coli* the protein was demonstrated to produce insoluble inclusion bodies. The proteins were solubilized in 8M urea and purified using nickel column chromatography using standard procedures. Following purification of the antigen we immunized mice and boosted them 21 days and 34 days later. Mice were immunized with 1 ug of antigen. Figure 5 demonstrates the immune response to antigen 1 following immunization in mice. Antibody levels begin rising at 21 days post vaccination and with boosting at Day 21 the responses increased dramatically. Boosting at Day 34 did not enhance immune response any further and, in fact, there was a gradual decline for the next three weeks. Similar responses were observed with antigen 2.

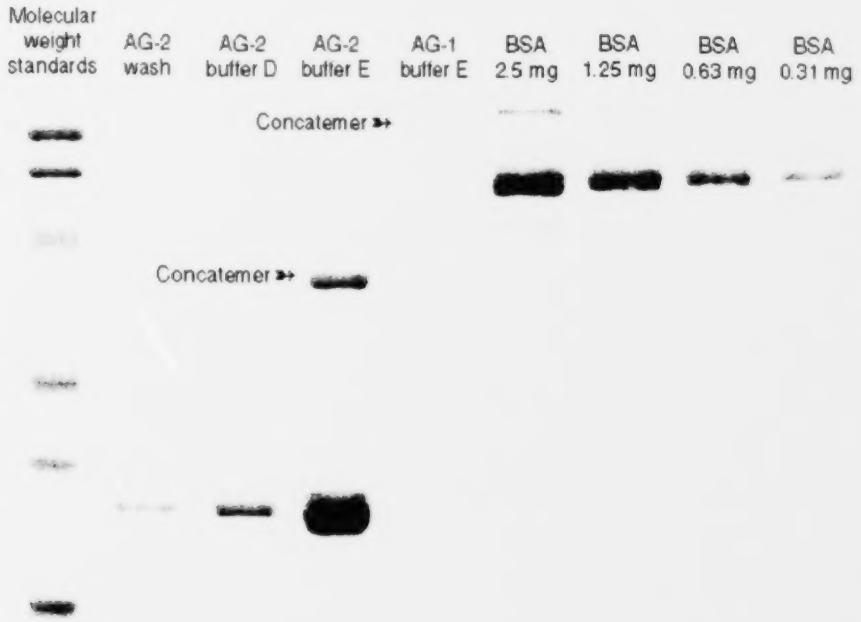
i) <u>Induction of immunity to Cryptosporidia antigens</u>

Since our eventual goal is to immunize cattle, one of the impediments to active immunization against Cryptosporidia is that calves generally get infected very early in life. Thus, there is minimal time to immunize calves at birth to protect them from Cryptosporidia one week after birth. Thus, the current vaccines for neonatal calf diarrhea are given to pregnant cows which then transfer high levels of maternal antibody to the calf as it suckles. This antibody bathes the intestine and prevents the attachment of the infectious agent to the intestinal cells. Thus, the vaccination is active immunization of the dam to passively protect the offspring. To simulate this approach, we immunized female mice and then collected the colostrum/milk from the pregnant animals. Antibodies to Cryptosporidia were undetectable in the immunized group of mice as expected. Thus, our mice colony does not have a natural Cryptosporidia infection. In contrast, the group immunized with antigen 1 had very high levels of antibody in the colostrum (Fig. 6). This antibody was transferred from the mothers to the stomachs of neonates. Interestingly, in this experiment we did not get any antibody responses to antigen 2, however, there appeared to be an enhanced immune response to the groups where the vaccine combined antigen 1 and 2. We need to repeat this experiment since we do not understand why in this specific experiment antigen 2 was not immunogenic.

In addition to testing for antibody, we also assessed the ability of antigen 1 and antigen 2 to induce cellular immune responses (Fig. 7 and 8). To achieve this, we used ELISPOT profiles for CD4T lymphocytes. Precursor frequencies of interferon gamma and IL-4 producing cells were significantly increased in the immunized animals as compared to the control animals. When immunized with AG-1 alone spleen CD4 positive T lymphocytes appeared to have a combined Th1 and Th2 response in their IL-4 precursor frequency was equal to that of interferon gamma when cells were stimulated with antigen 1 and greater than the control. When antigen 1 was combined with antigen 2 there was a significant increase in the number of T lymphocyte precursor cells as well as a shift to Th2 dominance with a significant rise in IL-4 precursor frequencies as compared to interferon gamma. Animals immunized with antigen 2 and antigen 1 or antigen 2 and stimulated with antigen 2 were TH2 dominant in that IL-4 precursor frequency was greater than interferon gamma. Again animals immunized with antigen 1 and antigen 2

have increased numbers of Th1 and Th2 T lymphocyte precursors than those immunized with antigen 2 alone (Figures 7 and 8).

In recent months we have been working to consolidate our earlier findings and put them on a firmer scientific foundation. We have shown that the genes for AG-1 and AG-2 contain previously unrecognized open reading frames. The molecular weights that these suggested did not always coincide with the proteins produced. It appears that these proteins spontaneously form chains (concatemers) of various molecular weights and that this is the reason for the discrepancy between the sizes of the open reading frames and the proteins. Nonetheless, all the proteins, regardless of size, are bound by the monoclonal antibodies. This shows that despite the formation of concatemers, the binding proteins are likely to remain suitable targets for our vaccine.



SDS-PAGE showing purification of the AG-1 and AG-2 antigenic proteins of *Cryptosporidium*

Most recently, we have performed histidine tagged purification of AG-1 and AG-2 proteins on a fairly large scale (See figure above). This will enable us to prepare vaccines in more practical quantities.

ii) <u>Additional Progress</u>

Although not a specific aim in the original grant, we have begun further expanding our research program in Cryptosporidia to develop methods to induce mucosal immunity to Cryptosporidium. We feel that induction of mucosal immunity will be critical for most effective control since this is a mucosal pathogen. Furthermore, enhancing mucosal immunity should also enhance lactogenic or colostral immunity, therefore, providing better protection to calves suckling immunized cows. In this regard, we constructed a

bovine adenovirus vector expressing one of the Cryptosporidia antigens. Using the full-length bovine adenovirus cloned genome, we inserted the gene encoding for the Cryptosporidia antigen and selected a variety of different plaques arising as a result of the transfection. One of the clones selected was BAV 364. Figure 9 demonstrates the expression of the Cryptosporidia antigen in BAV 364 at passage 1 and passage 10. This clone reacts very well to sera produced in a rabbit (02-121) that was immunized with a Cryptosporidia antigen. Figure 9 also demonstrates that the clone is stable even after 10 passages in culture with regard to expression of protein and by restriction enzyme analysis – a requirement for any vaccine development purposes. We have selected clone BAV-364 for further analysis and for future immunization studies of cattle. The levels of immune responses induced by a purified protein and by the adenovirus vector will be compared to select the most appropriate vaccine candidate.

Objectives 4 and 5: Vaccination with plasmid encoding AG2 or AG2 formulated with Quil A and CpG ODN

i) Construction of plasmids encoding AG1 and AG2 and *in vitro* expression of AG1 and AG2 in tissue culture.

The genes encoding AG1 and AG2 were inserted into pMASIA, which has reduced numbers of inhibitory motifs. This created pMASIA-AG1 and pMASIA-AG2 (Fig. 10). The recombinant plasmids were transformed into *E. coli* DH5α, purified using an EndoFree™ Plasmid Giga kit (Qiagen), and stored at -20 °C. *In vitro* expression of AG1 and AG2 in transfected Cos-7 cells was confirmed by immunohistochemistry. As shown in Fig. 11, both AG1 and AG2 are expressed from the respective plasmids *in vitro*.

ii) Immune responses induced by vaccination with plasmid encoding *Cryptosporidium* AG2 or pure AG2 formulated with Quil A and CpG ODN.

We subsequently performed an animal trial to compare the immune responses induced by plasmid encoding AG2 and purified AG2 protein formulated with Quil A and an immunostimulatory CpG containing oligodeoxynucleotide (ODN). We chose CpG ODN, because this compound has been shown to be an excellent Th1 stimulating adjuvant, and thus should induce strong cellular immunity, as well as humoral immunity. An experiment with AG1 protein is still in progress due to a delay in protein production. This was not part of the original objective, but an add-on.

Three groups of five ~~8 animals~~ mice were immunized two times with a four-week interval in the back with one of the following formulations: 1) Saline; 2) 20 μg AG2 formulated with 10 μg Quil A and 10 μg ODN 1826 or 3) 40 μg pMASIA-AG2. The plasmid was administered intradermally and the protein was delivered subcutaneously. At the end of the trial the humoral and cellular immune responses were determined by IgG ELISA and IFNγ and IL-4 ELISPOT assays. Fig. 12 shows that moderate antibody responses were induced by pMASIA-AG2, whereas AG2 with Quil A and CpG ODN elicited very strong antibody titers. In addition, significant numbers of IFNγ secreting cells were present in the animals of mice vaccinated with AG2 with Quil A and CpG ODN or pMASIA-AG2

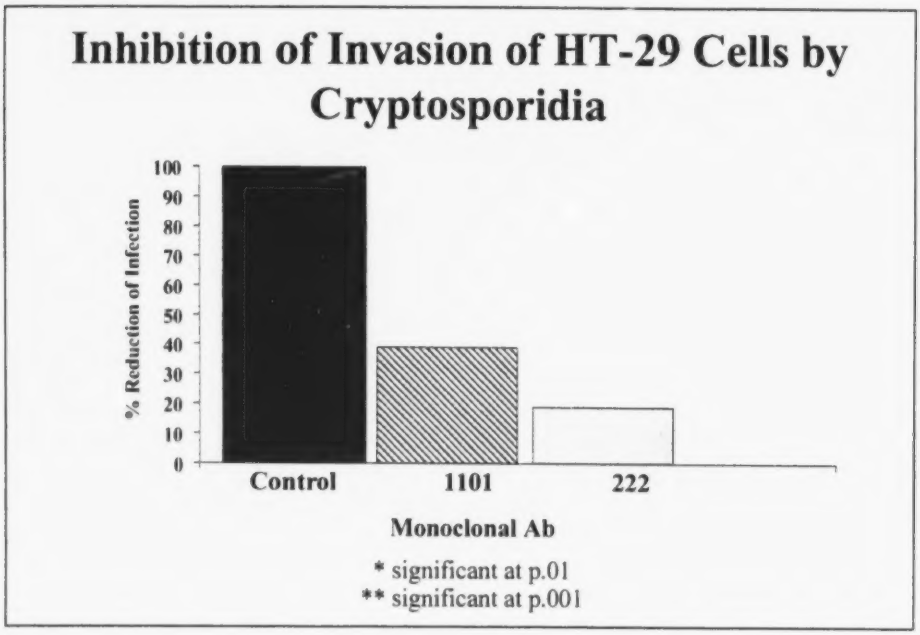
(Fig. 12), and there was no difference between the groups in this regard. The numbers of IL-4 secreting cells were lower than the numbers of IFNγ secreting cells in both vaccinated groups (Fig. 12), which suggests that all animals developed antibody and a Th1-type cellular immune response. These data demonstrate that DNA immunization resulted in strong cellular immune responses, whereas immunization with protein and CpG ODN and Quil A elicited strong cellular and humoral immunity.

Currently, we are challenging calves fed colostrums from vaccinated cows and seeing the level of protection of these calves following challenge with Cryptosporidium as compared to calves fed colostrums from unvaccinated cows.

Summary

We have completed all of the objectives as outlined and, indeed, have expanded the program to include both protein-based and DNA-based vaccines. DNA-based vaccines were the focus of the original objective. We have also developed an adenovirus vector vaccine (see previous progress report). Thus, we have three different vaccine candidates for head-to-head comparison which is currently being pursued, but was not part of the original proposal. The current and final progress report is describing the last objective which we had not completed in the previous reports indicating the immunization of animals with a DNA vaccine. Indeed, we are comparing the DNA vaccine with a protein-based antigen using novel formulations which we have developed.

Figure 1 – Inhibition of Cryptosporidia infection of HT-29 cells by monoclonal antibodies directed against Cryptosporidia proteins.



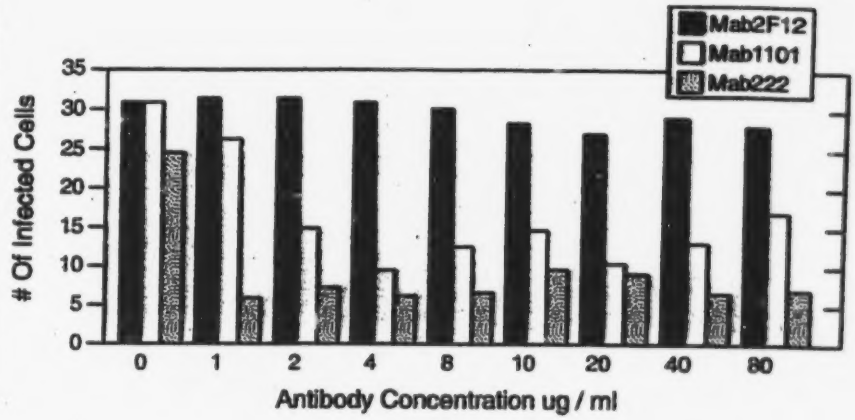


FIG 2a

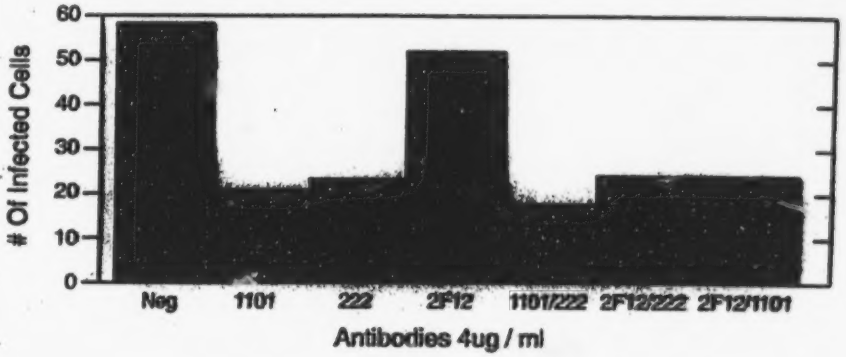


FIG. 2b

Figure 2 – Inhibition of invasion of Cryptosporidia of HT-29 cells. Different concentrations of monoclonal antibodies were tested individually (2a) or in combinations (2b).

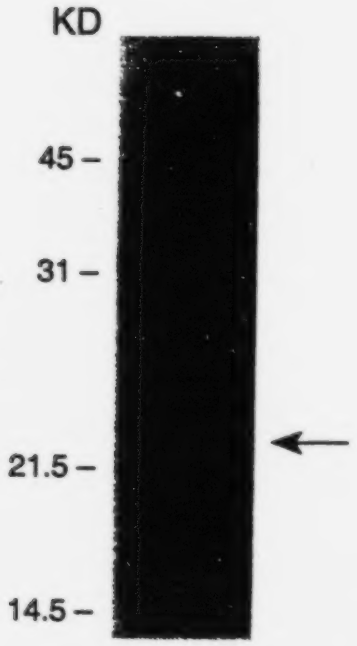


FIG._3

Figure 3 – Western blotting of Cryptosporidia protein with a monoclonal antibody to *Cryptosporidia parvum*.

ANTIGEN 1

```
        10         20         30         40         50         60         70         80         90        100
GAATTCGGCACGAGAATTACCATCTGATAGATCAAATTTACTTACATCTATTTTTACTACATTAAATATGGAGGAAAAACAGTCAATGAGCAATCCACAA
      A  R  E  L  P  S  D  R  S  N  L  L  T  S  I  F  T  T  L  N  M  E  E  K  Q  S  M  S  N  P  Q
                  5                 10                 15                 20                 25                 30

       110        120        130        140        150        160        170        180        190        200
TCGAAAAATACGAATACAAGCAATACCAACCACAAAGATTCTGGTTTAAATGATAAAATATTTGAAATGATTACAGATGAATTCAAAAAATTGACCTTTA
S  K  N  T  N  T  S  N  T  N  H  K  D  S  G  L  N  D  K  I  F  E  M  I  T  D  E  F  K  K  L  T  F  S
         35                 40                 45                 50                 55                 60                 6

       210        220        230        240        250        260        270        280        290        300
GCTTGTCCAAAGAATTAAATGATTCGGTTTCTTCAGCAATTAGCAAGTATTTAGAACCGATCGAACGTGATATACATCTATTAAGTCGCATTTGTCAGGA
  L  S  K  E  L  N  D  S  V  S  S  A  I  S  K  Y  L  E  P  I  E  R  D  I  H  L  L  S  R  I  C  Q  E
5                 70                 75                 80                 85                 90                 95

       310        320        330        340        350        360        370        380        390        400
ATCGAGAAGTCTGTTGATAATTATGTTAATATCAATGAAATTTCTAAAATTGAAACAAATGTTAAGGAACTTCTTACAAGTACAAATGAGAAATTAACAA
  S  R  S  L  L  I  I  N  L  I  S  M  K  F  L  K  L  K  Q  M  L  R  N  F  L  Q  V  Q  N  R  N
    100                105                110                115                120                125

       410        420        430        440        450        460        470        480        490        500
GCATCGACACTTGTATTTCGAGGCTTGTTGGCGAATCTAGAAGCGTTCGTGAAAAAGTGACTAAATTAAATAAACAATGCGATAACATTAACTCGAATCC

       510        520        530        540        550        560        570        580        590        600
AATAGACAACTTTACTCAAGTAGTAGCAGATTCATTTGGGACATTAACTAATGCAGTTACTCAATTGCAAACAACTGTTAATCGTTTGGAATTACAGATC

       610        620        630        640        650        660        670        680        690        700
AGTAATGGAATACCACTAAAAACGTCTTACACCAGATAACTCAATTACAATAAGAGCGCCCCAAAACATAGCTTTGCAAATTGATGATGCCTTAAACCAA

       710        720        730        740        750        760        770        780        790        800
AACATTACGATCGGCATTTCGGATAGCAATTCTGGATCAATCTAACTCTATCTCAATCAGATAAGAGAGAAATCCAAGCGGAGAATGTTTTGTTTTGAAC


       810        820        830        840        850        860        870        880        890        900
CTTGCATATAAGTTTGACCCATGTGTTTGGTTGGATGATCCAGCTTCCTATTAGCCATCCAGTAATATTAGGAATTTCAAAAATTTTGAGCGATACTCTT

       910        920        930        940        950        960        970        980        990       1000
CCTCATTTAATTGAATCTTTAAAGACCAGCTGTAATTTTTCTCAAATTAGCTGCATTTCAGATTTAAAATTGAGAATATTATGGATAAAAGAAACCATTC

      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
ACTGTTTTGAACCATTTACTGATACTCTTACACCTAGTGAGTAATGCAAATACTAAATGAAATTTCAGAAGTATGAACAAATGCATTAGCATTATAAATT

      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
CGGTTAGCGATGCAAGTAAAAGCATGAACTATATTGTACCTGCATGCTTCACGGATGGGTCGATCATCAGTGATATTACTAGCATATTAAGGCATATTAG

      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
AAGGACAACTAGAAATATTACATCTGGAATGAAATAAATTAATAAGGGGTAGAATTAGATATTTTTCATGTAAATAAATTAGCGTTATTGAGGATTATTC

      1310       1320       1330       1340       1350       1360       1370       1380
GAAATAAATAATAGAGATATTAAGTTTAGTTTTTATTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG
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1/9

2/9

Figure 4a - Gene sequence of Cryptosporidia antigen 1

ANTIGEN 2

```
        10         20         30         40         50         60         70         80         90        100
GAATTCGGCACGAGATTTTTTTTTTCTTTTACCTATTTCAATTAGTTTCTTTGATTCAAACGATGCAAAGTCATTATTTGTTTTAAATCCAGATGGATC
       H  E  I  F  F  F  L  L  P  I  S  I  S  F  F  D  S  N  D  A  K  S  L  F  V  L  N  P  D  G  S
                   5         10         15         20         25         30

       110        120        130        140        150        160        170        180        190        200
CGGAATTTTGAAAAACATTTCTACTAAATTCGAAATTAAATTTGAGCTTGGCTTGATAAATGGTAGTTGGCTCGGAGGTGATATTTTTATCCTTGATAGG
 G  I  L  K  N  I  S  T  K  F  E  I  K  F  E  L  G  L  I  N  G  S  W  L  G  G  D  I  F  I  L  D  R
          35         40         45         50         55         60

       210        220        230        240        250        260        270        280        290        300
AAACACGCTCTTGAAGCTGTAAGTTATTCAATCGCTTGTGTTTTCTATACAAAAACATGTTTTGAAAAGAATGAAGCACATTGTCTTAAACCCTTTAATC
K  H  A  L  E  A  V  S  Y  S  I  A  C  V  F  Y  T  K  T  C  F  E  K  N  E  A  H  C  L  K  P  F  N  R
65         70         75         80         85         90         95

       310        320        330        340        350        360        370        380        390        400
GCGCTGAGAATAAAATGACTTTTGGTTCTGAGAAAGACTTAGCGACAACTCTCCAATCTTCTAATTCTGAATATTATCTTTTCCTTACATGGAATAACTG
 A  E  N  K  M  T  F  G  S  E  K  D  L  A  T  T  L  Q  S  S  N  S  E  Y  Y  L  F  L  T  W  N  C
  100        105        110        115        120        125        130

       410        420        430        440        450        460        470        480        490        500
CATTCTTGGATATATTCCAATTAACACAAATAAAATCAACAAAATTTCTCTTGAAAGTTCCGGAGAAAACTCAATCTCCACAATTGGATATTGGAGTATT
 I  L  G  Y  I  P  I  N  T  N  K  I  N  K  I  S  L  E  S  S  G  E  N  S  I  S  T  I  G  Y  W  S  I
       135        140        145        150        155        160          1

       510        520        530        540        550        560        570        580        590        600
ATCGATGGATTTTCTTCTTCTTTAATTAAACATGCGCCTATAAAAGAAAATGGCCACTTGAATAATCAAGAATCAAAATATTCAAAATGAAATAATGAAG
 I  D  G  F  S  S  S  L  I  K  H  A  P  I  K  E  N  G  H  L  N  N  Q  E  S  K  Y  S  K
65         170        175        180        185        190

       610        620        630        640        650        660        670        680        690        700
CCACTAAACTCAACAAATCCAGAATCAGGTGGGAATAACTTAACTCAGAACCAAAACACAAAGCCTCATCCAGTTGTTAGACCGCATCCTACAGAAAAGC

       710        720        730        740        750        760        770        780        790        800
CCTCAAATGGTGAACATCAAGAATCTGGTTCAGAGCAAGCCCCTATTACCTCACCAGAAAACGAATCAAGTTCAAATCATCCTTCTGTGACAGTTCCAGA

       810        820        830        840        850        860        870        880        890        900
TACTGGATCAGTTCAAATCTCCTTCTGTTACTATTCCAGAGACTGGATCAGACTCAGATCACGCGCCTTGTGACAATTCCAGAGACTGGATCAGTTCAAA

       910        920        930        940        950        960        970        980        990       1000
TCATCTTCTGCTACTATACCAGAAACAGGATCCAGCTCAGATCACACTCTGCTACTTCTCCAGAAGAAGGATTGGACTCAGAACGTTACCAATCACTTCT

      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
ACAGAACAAACTCAAAGCCAGCTACATATCCTAACCAAGAAAATGAAAATCATAATAATCAGGAAGGTAATTCGAGTTTTAATACACTAAATCTTCCAAA

      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
TCAACCCAATCTTTCACGCAAGCTGGCAGATGTGGAAAGTTATGGGGAAAAGGATAAAATGGTTGATGGTGAGCAAGTAATCACTAAAAATGACATTATT

      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
GAAGATACTTCGAAAGAAATTAGAAACAAAATGTAAAGTATCTGCATTGATAAATATGGCCTTAGCCATTTCCAAATATCTAAATTGTCAACTCAAGTAA

      1310       1320
AAAAAAAAAAAAAAAAACTCGAG
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Figure 4b - Gene sequence of Cryptosporidia antigen 2

Figure 5

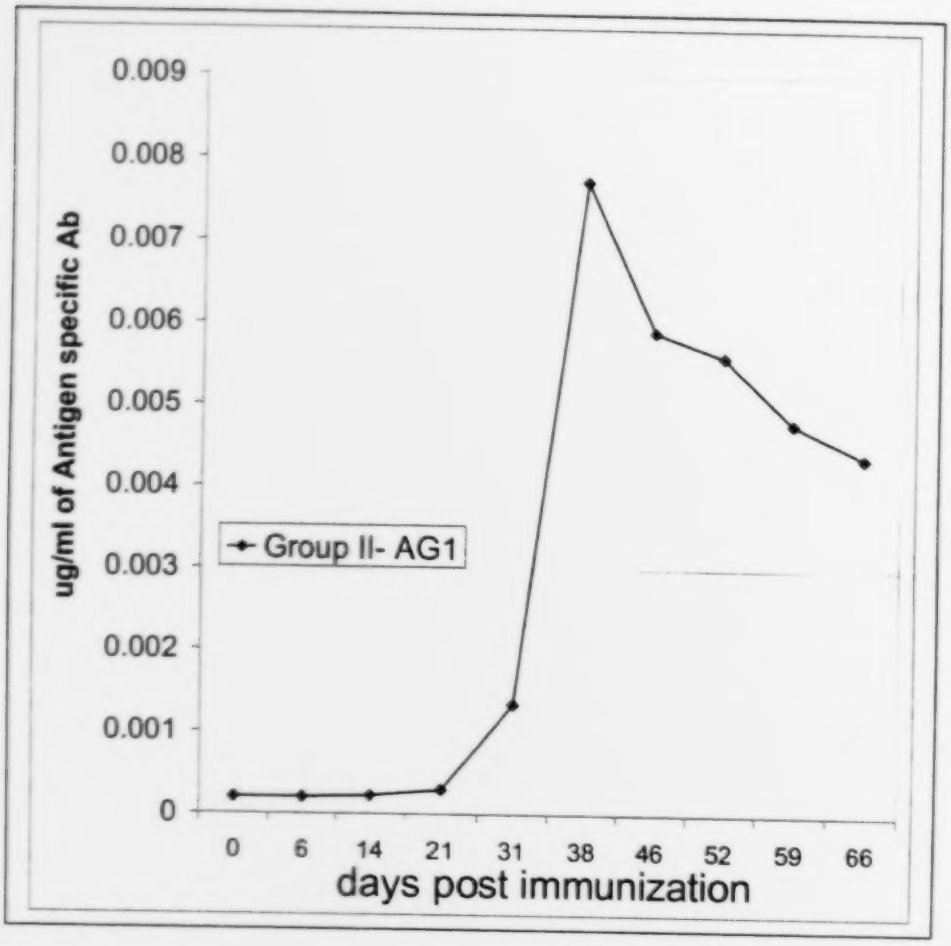

0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
ug/ml of Antigen specific Ab
Group II- AG1
0
6
14
21
31
38
46
52
59
66
days post immunization

Figure 6

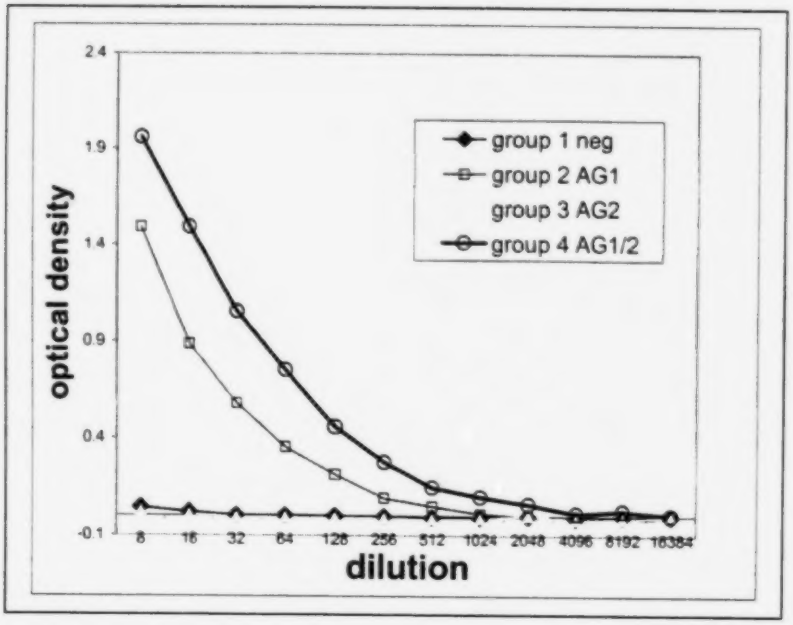


Figure 7

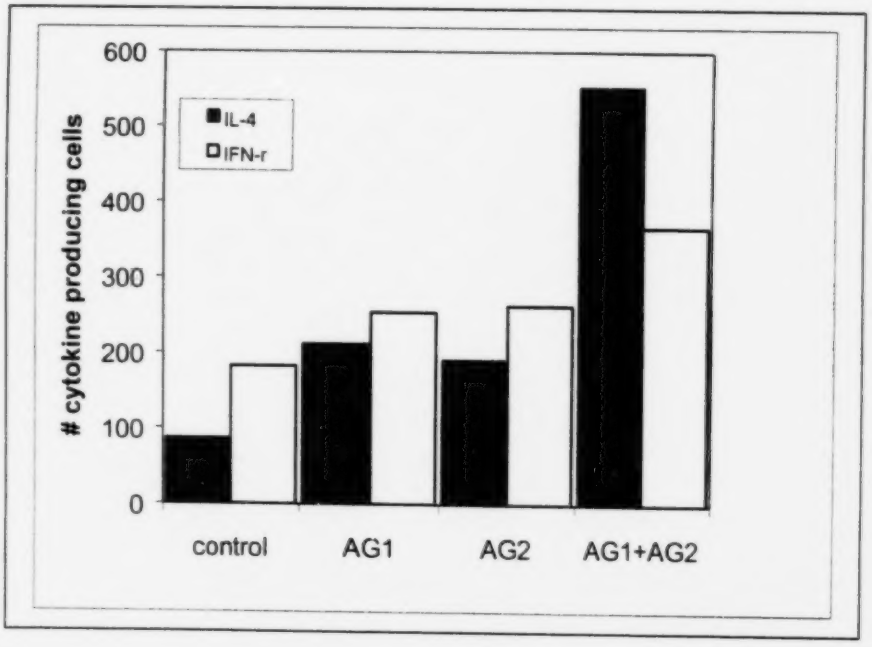


Figure 8

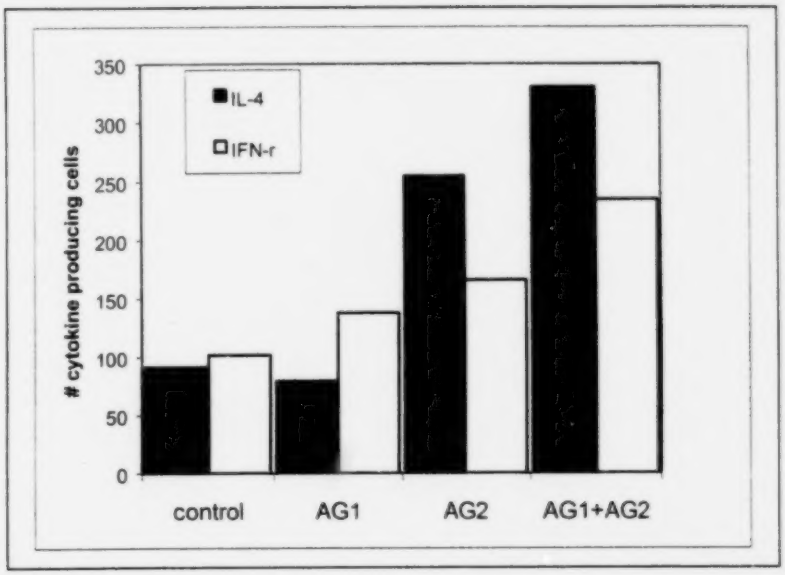
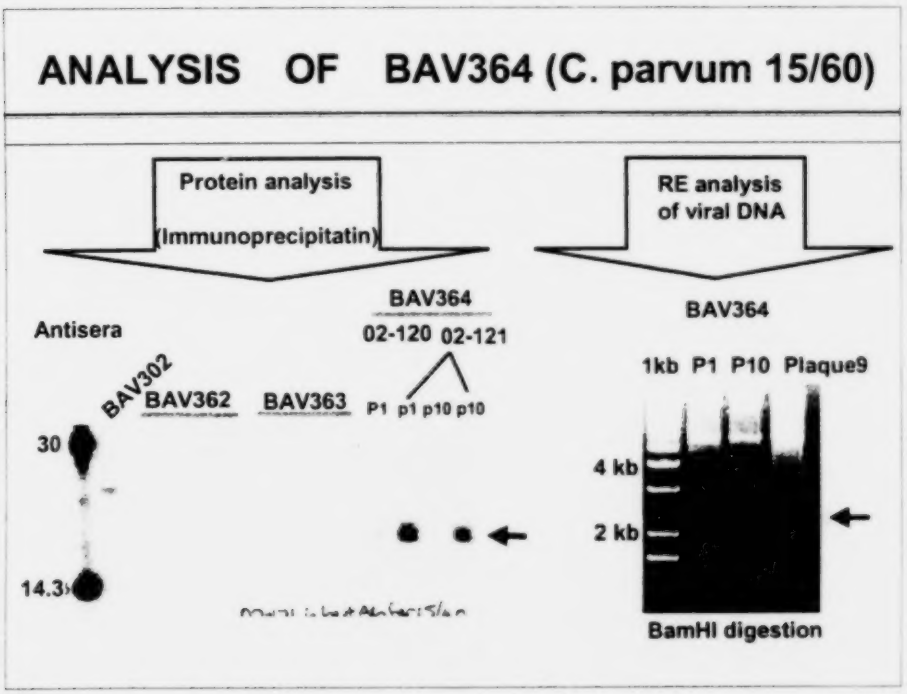
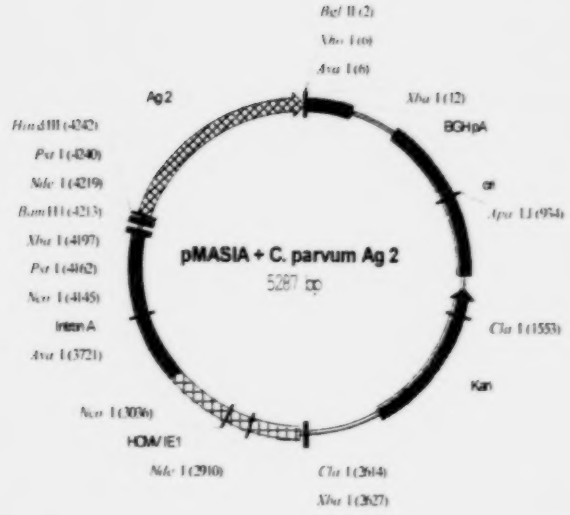


FIGURE 9





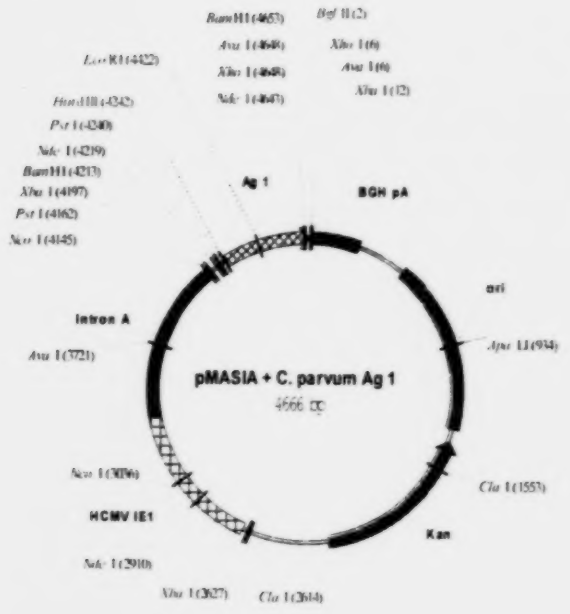


Figure 10 Plasmids encoding AG1 and AG2.

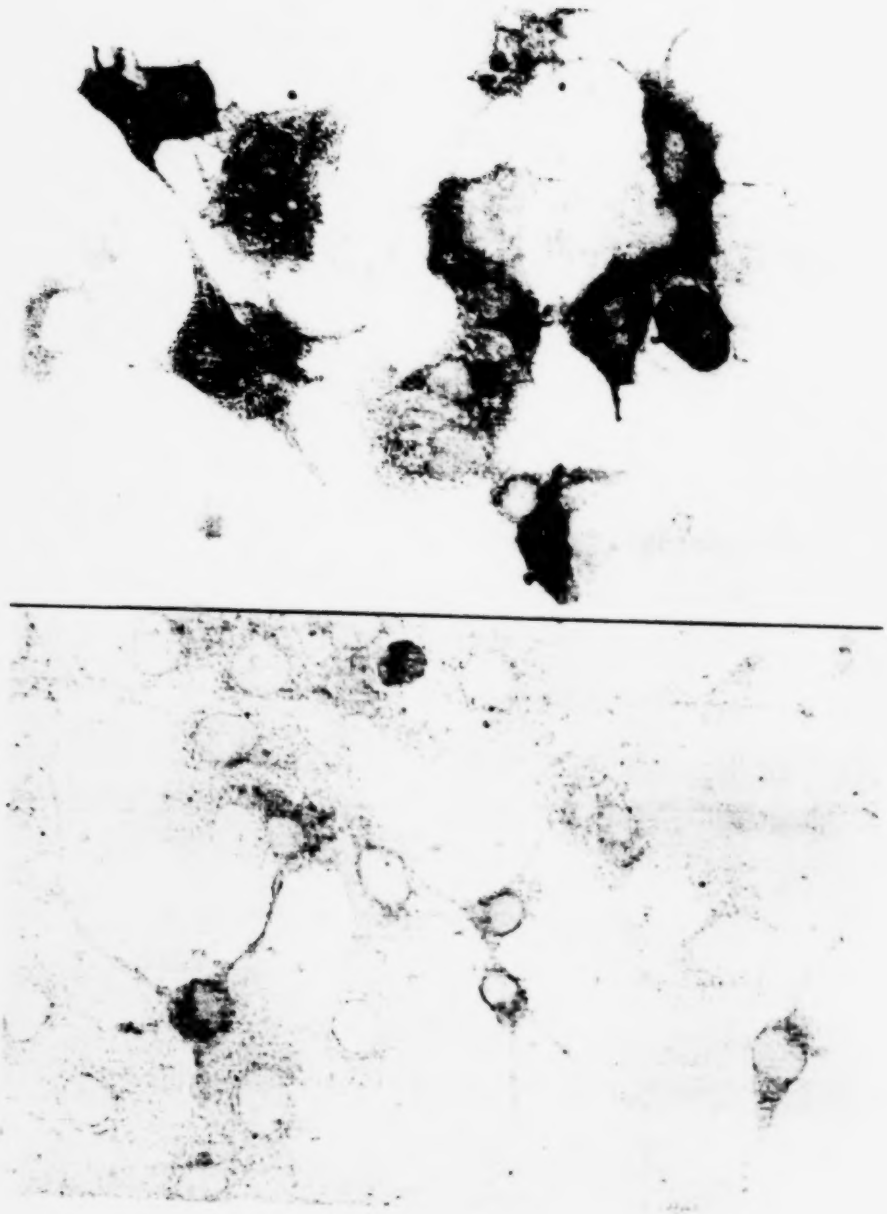


Figure 11. AG1 (top panel) and AG2 (bottom panel) expression identified by immunohistochemistry in COS-7 cells transiently transfected with pMASIA-AG1 or pMASIA-AG2. The presence of the antigens in the transfected cells was visualized with a specifc polyclonal antibody, followed by biotinylated horse anti-mouse IgG (Vector Laboratories) and a DAB substrate (Vector Laboratories).

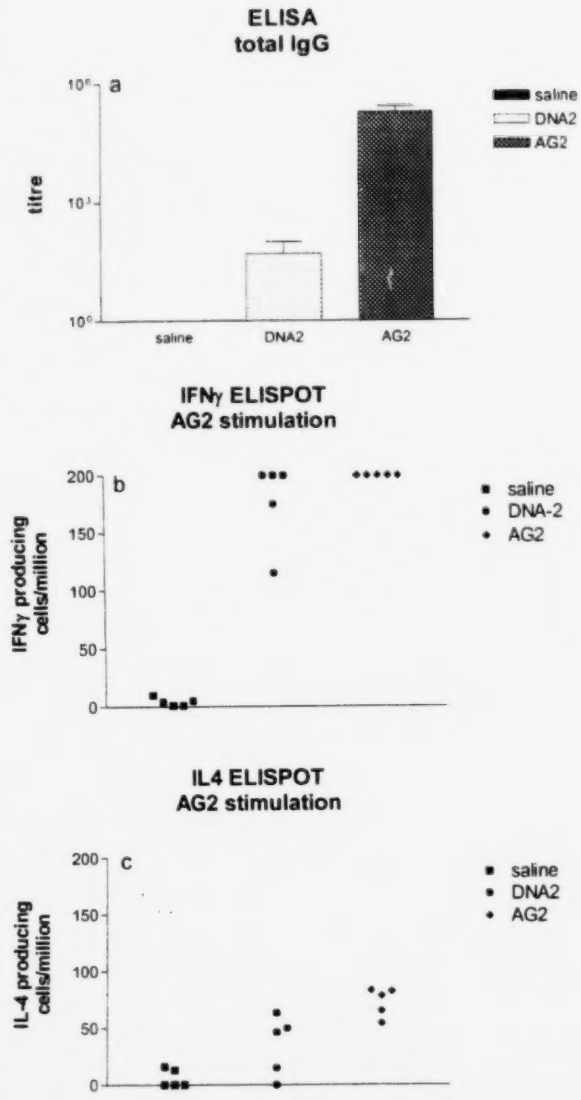


Figure 12. Immune responses of animals vaccinated two times with AG2 with Quil A and CpG ODN or pMASIA-AG2. **(a)** Total IgG in serum. Antigen-specific ELISA titres are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of negative control sera. Error bars show the SEM of five animals. **(b)** IFN-γ and **(c)** IL-4 secretion detected by ELISPOT assay. Lymphocytes were stimulated *in vitro* for 20 h with AG2. Differences in the number of cytokine secreting cells per 10^6 cells in antigen-stimulated wells and the number of cytokine secreting cells per 10^6 cells in non-stimulated wells are shown. The results represent the average value of triplicate wells.